Age-related endothelial dysfunction in human skeletal muscle feed arteries: The role of free radicals derived from mitochondria in the vasculature

Running Title: Mitochondria derived free radical in vasculature

Song-Young Park[1*], Oh Sung Kwon[2,3,*Ŧ], Robert H. I. Andtbacka[5], John R. Hyngstrom[5], Van Reese[2], Michael P. Murphy[6] and Russell S. Richardson[2,3,4,7]

[1]Department of Medicine and Whitaker Cardiovascular Institute, Boston University, Boston, MA, USA

[2]Geriatric Research, Education, and Clinical Center, George E. Whalen VA Medical Center, Salt Lake City, UT, USA

[3]Department of Internal Medicine, Division of Geriatrics, University of Utah, Salt Lake City, UT, USA

[4]Department of Exercise and Sport Science, University of Utah, Salt Lake City, UT, USA

[5]Department of Surgery, Huntsman Cancer Hospital, University of Utah, Salt Lake City, UT, USA

[6]MRC Mitochondrial Biology Unit, Cambridge Biomedical Campus, Cambridge, UK

[7]Department of Nutrition and Integrative Physiology, University of Utah, Salt Lake City, UT, USA

[*] These authors contributed equally to this work.

[Ŧ]Corresponding author:

Oh Sung Kwon
Physical address: George E. Whalen VA Medical Center,
Bldg 2, Rm 1D30, 500
Foothill Drive, Salt Lake City, UT 84148
Phone: (801) 582 1565 ext. 4344
E-mail: oh-sung.kwon@utah.edu

NEW & NOTEWORTHY

1. What is New?

• Free radicals from vascular mitochondria with advancing age play a critical role in attenuating NO bioavailability and, subsequently, promote endothelial dysfunction in the skeletal feed arteries (SMFAs) of the elderly.

• Mitochondria-targeted antioxidant, MitoQ, acutely restores SMFA endothelial function in the old to that of the young.

2. What is Relevant?

• Scavenging free radicals from within the mitochondria of the vasculature with mitochondria-targeted antioxidants reverses age-related vascular dysfunction which is a linked to cardiovascular disease.

3. Summary

• Mitochondria-targeted antioxidants, such as MitoQ, may be a useful pharmacological therapy in terms of counteracting the vascular dysfunction so often associated with advancing age and cardiovascular disease (CVD).

ABSTRACT

This study sought to determine the role of free radicals derived from mitochondria in the vasculature in the recognized age-related endothelial dysfunction of human skeletal muscle feed arteries (SMFAs). A total of 44 SMFAs were studied, 18 from young (32±6 yrs) subjects in control conditions and 26 from old (75±7 yrs) subjects with and without acute exposure to the mitochondria-targeted antioxidant MitoQ and nitric oxide synthase (NOS) blockade. The relative abundance of SMFA proteins from the electron transport chain (ETC), phosphorylated (p-) to endothelial (e) NOS ratio, manganese superoxide dismutase (MnSOD), and the mitochondria-derived superoxide (O_2^-) production were assessed. Endothelium-dependent and -independent SMFA vasodilation was assessed in response to flow-induced shear stress, acetylcholine (ACh), and sodium nitroprusside (SNP). The ETC proteins were lower in the old and were not altered by MitoQ. MitoQ restored endothelium-dependent vasodilation in the old to that of the young when stimulated by both flow (Young: 68±5; Old: 25±7; Old+MitoQ 65±9 %) and ACh (Young: 97±4; Old: 59±10; Old+MitoQ: 98±5 %), but did not alter, the initially uncompromised, endothelium-independent vasodilation (SNP). Compared to the young, MitoQ in the old attenuated the initially elevated mitochondria-derived O_2^- production and increased the initially attenuated level of MnSOD. Furthermore, MitoQ increased the ratio of p-eNOS/NOS and the restoration of endothelium-dependent vasodilation in the old by MitoQ was ablated by NOS blockade. Thus, free radicals derived from mitochondria in the vasculature of the elderly appear to play a critical role in attenuating NO bioavailability and, subsequently, endothelial dysfunction with advancing age. (Words 253)

ABBREVIATIONS LIST

SMFAs, skeletal muscle feed arteries; NOS, nitric oxide synthase; ETC, electron transport chain; MnSOD, manganese superoxide dismutase; O_2^-, superoxide; ACh, acetylcholine; SNP, sodium nitroprusside; NO, nitric oxide; TPP, triphenylphosphonium; L-NMMA, N^{ω}-nitro-L-arginine methyl ester; PSS, physiological saline solution; $ONOO^-$, peroxynitrite; CVD, cardiovascular disease

INTRODUCTION

With advancing age, blood flow to skeletal muscle is often diminished (24, 31), which, at least in part, is likely a consequence of attenuated endothelial function in the skeletal muscle resistance vasculature (9, 33, 34). However, the specific mechanism(s) responsible for the age-related attenuation of skeletal muscle blood flow is currently not well understood. The study of human SMFAs is highly germane to better understanding the vascular biology of aging, as it affords the opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood flow, also have regulatory potential (18). Indeed, our group has recently documented that the vasodilatory function of SMFAs obtained from elderly human subjects was markedly attenuated and this functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS protein levels (29). Although attenuated NO bioavailability with advancing age may depend on multiple factors that regulate NO production and degradation, free radicals, principally O_2^- (2, 14, 21), likely play an important role by reacting rapidly with NO, thereby decreasing NO bioavailability (21, 37). Currently, the exact source of the free radicals that appear to attenuate NO bioavailability and subsequent endothelial dysfunction with advancing age remain unclear.

Mitochondria play a critical role in cellular function in both health and disease, but are also an important and major source of free radicals (22, 35). Interestingly, although mitochondrial

content is relatively low in vascular endothelial cells and smooth muscle (2-5% of cell volume) compared to physically active skeletal muscle and cardiac myocytes (5-35 % of cell volume) (11), previous studies have revealed a strong correlation between mitochondria-derived oxidative stress and endothelial dysfunction (2, 8, 35). Interestingly, our group recently documented that exercise training induces an increase in vascular mitochondrial respiratory capacity, evidence of improved redox balance, and elevated basal NO bioavailability (30). These data suggest that age- and disease-related alterations in arterial function may be directly affected by the function, and subsequent free radical production, of mitochondria in the vasculature. Therefore, strategies to constrain mitochondria-derived free radical levels to within typical physiological levels may prove useful in attenuating the development of endothelial dysfunction with age.

The first line of defense against free radicals is both endogenous and exogenous antioxidants. However, to date, antioxidant supplementation (e.g. Vitamin C) has not proven effective at specifically decreasing mitochondria-derived free radical production (1, 19). Of note, as mitochondria are negatively charged, the incorporation of a lipophilic cation, such as triphenylphosphonium (TPP), to a potent antioxidant, such as the active ubiquinol moiety of Coenzyme Q10, enables the selective and extensive accumulation of the antioxidant within the mitochondria (26, 27). Utilizing this approach, a commercially available mitochondria-targeted antioxidant, MitoQ (MitoQ Limited, Auckland, NZ), has been synthesized to yield a thousand-fold greater concentration within the mitochondria than untargeted antioxidants, which distribute throughout the cell (26, 27). The use of MitoQ to specifically treat age-related endothelial function is supported by a recent, elegant and comprehensive, study by Gioscia-Ryan et al., (15) who reported that this mitochondria-targeted antioxidant attenuated endothelial dysfunction in

older mice. Nevertheless, age-related vascular mitochondrial free radical production and endothelial dysfunction in humans has yet to be examined.

Consequently, utilizing the pressure myography technique and incubation with MitoQ, this study sought to determine the role of free radicals derived from vascular mitochondria in the age-related endothelial dysfunction of human SMFAs. We tested the hypothesis that free radicals derived from vascular mitochondria play a critical role in attenuating NO bioavailability and, subsequently, promote endothelial dysfunction in the elderly.

METHODS

Subjects and general procedures: A total of 44 SMFAs were obtained from young and old subjects, from the axillary and inguinal regions, during melanoma-related surgeries. From these SMFAs, endothelial-dependent and -independent vascular function was assessed in 10 young subjects, while 16 old subjects were assessed with and without MitoQ. A subset of these vessels (n = 8 young and 8 old subjects) were assessed for mitochondria-specific O_2^- production. Endothelial-dependent vascular function was assessed in the SMFAs from the remaining 8 young subjects, while the remaining 10 old subjects were assessed with and without MitoQ and N^{ω}-nitro-L-arginine methyl ester (L-NMMA). Unused segments of these vessels (n = 8 young and 10 old subjects) were used for immunoblotting. It should be noted that, although all subjects were free from cancer and chemotherapy, there were no other specific exclusion criteria for this study. However, all medical conditions and medications were noted. All protocols were approved by the Institutional Review Boards of the University of Utah and Salt Lake City Veteran's Affairs Medical Center (VAMC), carried out in accordance with the Declaration of Helsinki, and written informed consent was obtained from all subjects prior to surgery.

Vessel harvest and preparation: SMFAs (outer diameter ~500 μm, length 1-2 cm) from the axillary (e.g. serratus anterior or latissimus dorsi muscles) and inguinal (e.g. hip adductors or quadriceps femoris muscles) regions, obtained during sentinel node biopsy for melanoma surgery at the Huntsman Cancer Hospital and the Salt Lake City VAMC, were studied. Patients were anaesthetized using a general protocol: propofol, fentanyl, benzodiazepines, and succinylcholine (28). SMFAs were harvested during dissection to locate sentinel lymph nodes, for clinical analysis, and were identified and classified based upon being a vascular inlet into a muscle bed, structure, coloration, and pulsatile bleed pattern (17). SMFAs were ligated, excised, and immediately placed in iced normal physiological saline solution (PSS) before being transferred to the laboratory within 15 min of harvesting (29).

MitoQ treatment and vessel function protocols: Initially, perivascular adipose and/or connective tissue around the SMFAs was removed under a dissecting microscope (SZX10; Olympus, Center Valley, PA, USA) in cold (4 °C) PSS containing (mM): 145.0 NaCl, 4.7 KCL, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS buffer and 1 g $(100\ mL)^{-1}$ BSA at pH 7.4. SMFA function was assessed in pressure myography organ baths (110p; DMT Systems, Aarhus, Denmark) (29). The arteries were cannulated at both ends with micropipette tips and then pre-incubated for 30 min within the bath in either PSS, the control condition, or MitoQ mesylate (10 μM). After the pre-incubation period, the vessel outer diameters were recorded using an inverted microscope with a video camera (TS100; Nikon Eclipse, Melville, NY, USA), with data streamed in real time to edge detection software (DMT VAS v 0.2.0), monitored at a sampling rate of 1 kHz. Fluid leak was detected by pressurizing the vessel to an intraluminal pressure set of 60 mmHg, closing the cannulas to the fluid reservoirs, and assessing any change in vessel diameter. Arteries, free from leaks were then warmed to 37°C,

allowed to develop spontaneous tone for a 30 min equilibration period, and then vasodilatory function was assessed (29).

Vasodilation assessments: Vasodilatory dose response curves (%) were assessed for three stimuli: First, to assess the endothelium-dependent vasodilatory response to flow-induced shear stress, intraluminal flow was developed. This was achieved by altering the heights of the independent fluid reservoirs, contiguous with both cannulated ends of the SMFAs, in equal and opposite directions so that a pressure difference was developed across the vessel without altering mean intraluminal pressure. Three pressure differences of 15, 30, and 45 mmHg, which yielded an approximate flow rate of 15, 30 and 45 μl/min, were utilized for the flow experiments. Second, to assess endothelium-dependent vasodilation pharmacologically, an ACh dose response curve (ACh, 10^{-7} to 10^{-3} M) was performed following pre-constriction with phenylephrine (PE) (10^{-6} to 10^{-4} M) to ~70 % of the maximum PE response. Third, to assess endothelium-independent vasodilation, a SNP dose response curve was performed (10^{-9} to 10^{-4} M) following pre-constriction with PE (10^{-6} to 10^{-4} M) to ~70 % of the maximum PE response.

Mitochondria-specific O_2^- measurements: Mitochondria-specific O_2^- measurements were performed with EPR spectroscopy on the initially frozen SMFA segments using an EMX X-band spectrometer (Bruker, MA). Briefly, the segment of the frozen SMFA was placed into a micro centrifuge tube containing 150 μL of the mitochondria-specific O_2^- spin trap mitoTempo-H (Enzo Life Sciences San Diego, CA) (1-hydroxy-4 [2-(triphenylphosphoino) – acetamido] - 2,2,6,6-tetramethylpiperidine) (0.5 mmol/L) and incubated for 60 minutes at 37 ºC, facilitating the “thaw and trap” approach (10, 32). The samples were then placed on ice and 50 μL of the solution was loaded into a capillary tube for EPR spectroscopy analysis. The EPR spectroscopy

scan was run with a center field at approximately g = 2.004 and the area under the curve of the spectra was calculated by double integration (29).

Percent vasodilation calculations: Percent vasodilation was used for data expression to account for baseline differences in vessel diameter, and calculated using the following equation:

$$(DT\text{-}Dp/Di\text{-}Dp)\times 100$$

Where DT is the recorded diameter at a given time point, Dp is the diameter recorded after the addition of the vasoactive agent (i.e. pre-constriction diameter), and Di is the diameter recorded immediately before the addition of the vasoactive agent (initial diameter).

Immunoblotting: The relative abundance of proteins for the ETC complexes, p- and eNOS, and MnSOD were determined in SMFAs using Western blot analysis. Briefly, SMFAs were homogenized in lysis buffer, supplemented with a protease/phosphate inhibitor cocktail (10 μM sodium fluoride and 1 mM phenyl methyl sulfonyl fluoride (PMSF)) (Santa Cruz Biotech, Santa Cruz, CA). Protein concentration was determined using the Bradford technique. 50 μg of homogenate was separated by polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and incubated with primary and secondary antibodies directed against the proteins of interest. Membranes were imaged on a ChemiDoc XRS (Bio-Rad, Hercules, CA) and quantified with Image Lab software (Bio-Rad). The specific antibodies used to detect SMFA proteins included: Total OXPHOS Human Western Blot Antibody Cocktail (ab110411, Abcam, Cambridge, MA), total eNOS (610296, BD Transduction, San Jose, CA), p-eNOS at Ser1177 (9570, Cell Signaling, Boston, MA), and superoxide dismutase 2 (SOD2) (SC-515068, Santa Cruz Biotech, Santa Cruz, CA). The abundance of each protein was normalized to beta-actin (ab8227, Abcam, Cambridge, MA), which served as a loading control.

***Statistical Analyses*:** The statistical analyses were performed using GraphPad Prism 7 Software (La Jolla, CA). Two-way repeated measures ANOVA was used to assess changes in vessel diameter with and without MitoQ in response to flow, ACh, and SNP. Two-way repeated measures ANOVA were used to assess changes in vessel diameter with and without MitoQ and with and without L-NMMA in response to flow and ACh. When necessary, a Tukey's post hoc test was used to identify significant differences. For all other comparisons, one-way ANOVA was used to assess the group and, if necessary, a Tukey's post hoc test was used to identify the significant differences. For all analyses, a p-value of < 0.05 was considered significantly different. All data are expressed as mean ± SEM.

RESULTS

Subject characteristics: From the 44 SMFAs that were harvested, 18 were from young subjects (33±2 yrs) and 26 were from old subjects (72±5 yrs). The subject characteristics, obtained from preoperative examination of medical records, are presented in Table 1. Note that users of cancer-related medications were excluded from the study. Also, it should be noted that all blood chemistry and complete blood count results (Table1) were within normal ranges, suggesting that the subjects who participated in this study were relatively healthy.

Vessel characteristics: SMFAs were harvested from either the inguinal (n=23) or axial (n=21) regions from either males (n=25) or females (n=19). In agreement with our previous observations, vessel function was not different as a consequence of anatomic origin or sex. Immunoblotting, to assess the relative abundance of proteins in the ETC, revealed that the majority of the mitochondrial respiratory complexes, with the exception of Complex V, were significantly attenuated in the SMFAs of the old compared to the young (Figure 1). MitoQ did not alter this attenuation of the mitochondrial respiratory complexes in the old (Figure 1). Basal, unpressurized, outer diameter of the SMFAs was not statistically different in the young, old, and old with MitoQ (Young: 510 ± 12 *u*m; Old: 514 ± 15 *u*m; Old+MitoQ: 515 ± 10 *u*m). Additionally, maximal outer diameter of the SMFAs, achieved by Ca^{2+} free NPSS incubation, was not statistically different in the young, old, and old with MitoQ (Young: 758 ± 19 *u*m; Old: 752 ± 14 *u*m; Old+MitoQ: 750 ± 15 *u*m).

The vasodilatory response to flow, ACh, and SNP and the impact of MitoQ in the old: The PE-induced pre-constriction of the SMFAs prior to the flow stimulus was similar between groups (Young: 69 ± 4 %, Old: 67 ± 5 %, Old+MitoQ: 68 ± 5, $P > 0.05$). The greatest vasodilation in

response to the intraluminal flow of 45 ± 3 ul/min was significantly attenuated in the old compared to the young (Young: 68 ± 5; Old: 25 ± 7%, $P < 0.05$) (Figure 2A). However, the vasodilatory response to flow in the old was restored to that of the young by MitoQ (Old+MitoQ: 65 ± 9 %) (Figure 2A). This effect of MitoQ in the old was also evident at the lower intraluminal flow rates of 15 ± 2 and 30 ± 4 µl/min (Figure 2A).

The PE-induced pre-constriction of the SMFAs prior to the ACh and SNP dose response curves were similar between groups (Young: 69 ± 4 %*;* Old Control: 68 ± 5 %; Old + MitoQ 69 ± 5 %, $P > 0.05$). The greatest vasodilation in response to the highest dose of ACh (10^{-3} M) was significantly attenuated in the old compared to the young ACh (Young: 97±4 %; Old: 59±10 %, $P < 0.05$) (Figure 2B). However, the vasodilatory response to ACh in the old was restored to that of the young by MitoQ (Old+MitoQ: 98±5 %) (Figure 2B). This effect of MitoQ in the old was clearly evident across the whole ACh dose response curve (Figure 2B). In contrast, endothelial-independent vasodilatory function, the vasodilatory response to the highest dose of SNP (10^{-4} M) (Young: 97 ± 4 %, Old: 100 ± 11 %; Old+MitoQ: 98 ± 4 %, $P > 0.05$) and across the whole dose response curve, was similar among the young, old, and old with MitoQ (Figures 2C).

Levels of mitochondria-specific O_2^- and MnSOD and the impact of MitoQ in the old: The baseline EPR spectroscopy signal for the mitoTempo-H adduct in the SMFAs, an index of mitochondria-specific O_2^- production, was greater in the old compared to the young (Young: 1.7 ± 0.2; Old: 6 ± 1.8; AUC/mg, $P < 0.05$) (Figures 3A). However, MitoQ significantly lowered SMFA O_2^- production in the old, such that the old were similar to the youg (Old+MitoQ: 1.95 ± 0.7; AUC/mg) (Figure 3A). In terms of antioxidant status, immunoblotting revealed that baseline MnSOD protein content was significantly attenuated in the old compared to the young (Young: 100 ± 18; Old: 38 ± 17 AUC, $P < 0.05$) (Figure 3B). However, incubation with MitoQ

286 significantly increased the MnSOD protein content of the old (Old+MitoQ: 78 ± 15 AUC)
287 (Figure 3B).

288 **The role of NO bioavailability and the impact of MitoQ in the old:** Immunoblotting revealed
289 that the extent of eNOS phosphorylation, measured as the p-eNOS/eNOS ratio on the Western
290 blots, was significantly lower in the old compared to the young (Young: 100 ± 16; Old: 35 ± 18;
291 AUC $P < 0.05$). However, MitoQ enhanced the extent of eNOS phosphorylation in the old
292 (Old+MitoQ: 59 ± 18 AUC) (Figure 4). SMFA vasodilation, in response to both flow and
293 increasing doses of ACh, again revealed attenuated endothelial-dependent vasodilation in the old
294 which could be restored acutely by MitoQ (Figure 5A and B). However, the impact of the MitoQ
295 was negated by NOS blockade (Figure 5A and B). Furthermore, in the presence of L-NMMA the
296 vasodilatory response to both flow and ACh with and without MitoQ was attenuated to a level
297 that was significantly lower than the initial dose response in the old (Figure 5A and B).

298

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DISCUSSION

This study sought to determine the role of free radicals derived from mitochondria in the vasculature in the age-related endothelial dysfunction documented in human SMFAs. The main hypothesis tested by this investigation was that free radicals derived from aging vascular mitochondria play a critical role in attenuating NO bioavailability and, subsequently, promote endothelial dysfunction in the elderly. The current findings strongly support this postulate and, of importance, translate previous findings in an animal model to humans. Specifically, despite the observation that the ETC proteins were lower in the old, and this was not altered by MitoQ, this mitochondria-targeted antioxidant acutely restored SMFA endothelium-dependent vasodilation, in response to both flow and ACh, to that of the young. Additionally, MitoQ attenuated mitochondria-derived O_2^- production, likely sparing MnSOD, which resulted in an increase in MnSOD levels. Furthermore, in the old, the restoration of SMFA endothelium-dependent vasodilation by MitoQ was ablated by NOS blockade, and MitoQ increased the extent of eNOS phosphorylation. Thus, augmented mitochondrial free radical production in the SMFAs of the elderly appears to play a critical role in attenuating NO bioavailability and, subsequently, promoting endothelial dysfunction with advancing age.

Vascular aging, SMFAs, free radicals, and NO bioavailability:

In terms of the vascular biology of aging, the study of human SMFAs is pertinent, as it affords the opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood flow, also have regulatory potential (18). In fact, our group recently documented that the endothelial function of SMFAs attained from the elderly was markedly attenuated and this functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS protein

level, emphasizing the likely role of attenuated NO bioavailability (29). Here, the findings of this previous work were confirmed with further evidence that aging similarly attenuates both flow- and ACh-mediated vasodilation in SMFAs (Figure 2A and B), each indicators of endothelium-dependent vasodilation. The current findings further suggest that this limited vasodilatory capacity with advancing age is, at least in part, due to attenuated NO bioavailability, as again evidenced by a decrease in the ratio of p-eNOS to total eNOS protein expression in the SMFAs from the old (29) (Figure 4). Attenuated NO bioavailability with advancing age depends on multiple factors that regulate NO production and degradation, with a key role being played by free radicals. For example, O_2^- decreases NO bioavailability (2, 14, 21) by rapidly reacting with NO to form peroxynitrite ($ONOO^-$), but then, in turn, $ONOO^-$ may oxidise the essential co-factor for eNOS, tetrahydrobiopterin, resulting in O_2^- production, rather than NO, by eNOS (21, 37). This redox imbalance likely plays an important role in the age-related fall in NO bioavailability, supported in this study by the greater mitochondria-derived O_2^- production and reciprocally attenuated MnSOD levels in the old SMFAs (Figure 3A and B). Indeed, there is accumulating evidence that increased free radical production leads to endothelial dysfunction with advancing age both in animals and humans, and that the resultant oxidative stress promotes vascular disease (7, 25, 39).

MitoQ, age-related vascular dysfunction, and NO bioavailability:

The acute 1 hr incubation of the SMFAs from the old with MitoQ effectively reversed the age-related vascular dysfunction (Figure 2A and B). Several lines of evidence from this study suggest that this restoration of vascular function in the old SMFAs was NO mediated. First, MitoQ greatly attenuated mitochondrial O_2^- production to more closely resemble that of the young (Figure 3A), a change that would likely result in an increase in NO bioavailability. Again, it is

interesting to note that this fall in O_2^- production was accompanied by an increase in MnSOD (Figure 3B). This makes intuitive sense and suggests a MitoQ-induced sparing of this endogenous antioxidant that targets O_2^- and is found predominantly within the mitochondria. Second, MitoQ significantly increased the attenuated ratio of p-eNOS to total eNOS protein expression in the SMFAs from the old (Figure 4), indicative of rescuing the activity of this NO producing pathway. Third, the reversal of the age-related vascular dysfunction achieved by MitoQ during both the flow and ACh dose response curves was ablated by NOS blockade, confirming a role for NOS in the MitoQ-induced response. Furthermore, the flow and ACh responses with and without MitoQ, in combination with NOS blockade, were significantly attenuated compared to the flow and ACh assessments in the old SMFAs. Overall, this indicates that NO still plays a role in the response of the old vessels, but, more importantly, that MitoQ was ineffectual when NOS was blocked, implying an NO-mediated mechanism of action (Figure 2A and B). Although performed in stroke-prone hypertensive rats, the conclusion by Graham et al. (16) that MitoQ supplementation, initiated prior to the establishment of cardiovascular disease (CVD) in young animals, prevented the development of endothelial dysfunction by maintaining NO bioavailability, is in agreement with the premise of the current findings.

Vascular aging, SMFAs, blood flow, and oxygen transport:

It is widely accepted that aging is commonly associated with impaired blood flow, and subsequently oxygen delivery, to skeletal muscle during dynamic exercise and that this is likely caused by a combination of compromised cardiac output (17, 23) and attenuated peripheral vascular conductance with age (23, 25). In terms of the skeletal muscle vasculature, in rodent studies, the rate of endothelium-dependent vasodilation in the skeletal muscle arterioles, which are downstream from the SMFAs, and microcirculatory blood flow was attenuated in old

compared to young animals (3, 4), subsequently impairing oxygen delivery to the contracting muscles. In humans, our group recently provided evidence supporting the contention that human SMFAs, the inlets to the muscle bed upstream of the arterioles, regulate vascular resistance, and therefore skeletal muscle perfusion, in response to shear stress and pharmacological vasodilators (17, 29). Furthermore, our group has also demonstrated that SMFAs from older humans exhibit an attenuated magnitude of endothelium-dependent vasodilation and delayed vasodilation kinetics in response to shear stress and ACh (29). In agreement with these prior results, the current findings confirm that the endothelium-dependent vasodilatory capacity of SMFAs, assessed by flow-induced shear stress and the response to ACh, is clearly attenuated with advancing age (Figure 2A and B). This attenuated SMFA vasodilation with aging is likely one of the mechanisms responsible for the age-related decline in blood flow and oxygen transport to active skeletal muscle during physical activity in the elderly. In light of the current positive findings with MitoQ and the positive impact on age-related vascular function, additional studies examining the effect of mitochondria-targeted antioxidants on skeletal muscle blood flow during exercise in the elderly are warranted.

Mitochondrial health, vascular aging, and MitoQ

As the major energy producers for most physiologic processes, well-functioning, healthy, mitochondria are essential for both systemic and cellular homeostasis. However, in addition to a central role in energy production, mitochondria seem to be important in terms of molecular signaling and cellular secretion in the vasculature and this is mediated, at least to some extent, by free radicals (8, 11). Indeed, free radicals, produced at numerous sites within the mitochondria, including Complexes I, II, and III of the ETC, play a critical role in these processes. For example, it has been documented that mitochondria located in the endothelial cytoskeleton of arterioles, in

391 the human myocardium, produce free radicals in response to shear stress induced cell
392 deformation, which are critical for flow-mediated dilation (20). Conversely, several recent
393 studies have also revealed that mitochondria-derived free radicals in the vasculature play a
394 critical role in peripheral vascular dysfunction with advancing age (15, 36, 38). Interestingly, and
395 along these lines, both hyperglycemia and elevated triglycerides, recognized as inducers of
396 endothelial dysfunction and atherosclerosis, increase mitochondria-derived free radicals and alter
397 mitochondrial dynamics in vascular endothelial cells. This vascular dysfunction can be reversed
398 by normalizing the blood sugar and lipid load, removing the mitochondrial stimulus (6).
399 Furthermore, and perhaps somewhat ironically, in terms of mitochondrial health, mitochondria-
400 derived free radicals lower the abundance of MnSOD, which resides in the mitochondrial matrix,
401 and negatively impacts mitochondrial biogenesis and mitochondrial content (15).

402 The initial age-related findings from this study support the link between attenuated vascular
403 function with advancing age (Figure 2A and B) and compromised vascular mitochondrial health,
404 as evidenced by the greater O_2^- production (Figure 3A), lower levels of MnSOD (Figure 3B), and
405 the attenuation of the ETC complexes (Figure 1) in the SMFAs from the old. Interestingly, in
406 addition to restoring endothelial function in the SMFAs from the old (Figure 2A and B), the
407 acute 1 hour incubation with MitoQ both decreased mitochondrial O_2^- production (Figure 3A)
408 and restored mitochondrial antioxidant capacity (MnSOD) (Figure 3B). However, MitoQ did not
409 impact the relative abundance of ETC complex proteins (Figure 1). This is of particular
410 relevance in light of recent studies that have suggested aging is associated with attenuated
411 mitochondrial respiratory complexes (12) and that elevated mitochondria-derived free radical
412 production damages the mitochondrial DNA that encodes the ETC complexes (5, 13). This
413 damage, predominantly at complex I, appears to directly affect electron transport and disrupts the

whole mitochondrial respiratory cycle (5, 13). In the current study, although perhaps not surprising, due to the relatively short time course of the MitoQ exposure, the lack of effect on the significantly attenuated ETC complex protein expression is an important observation. Specifically, this documents that the positive impact of MitoQ on vessel function and mitochondrial free radical production is not dependent upon more long-term changes in the relative abundance of the mitochondrial complexes.

Conclusion

This study has demonstrated that, in human SMFAs, recognized to have regulatory potential, the attenuation of free radicals from the mitochondria in the vasculature, with a mitochondria-targeted antioxidant, reverses age-related vascular dysfunction by what appears to be an NO-dependent mechanism. These findings suggest that mitochondria-targeted antioxidants, such as MitoQ, may have utility in terms of counteracting the attenuated skeletal muscle blood flow and vascular dysfunction so often associated with advancing age and cardiovascular disease.

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Competing interests

M. P. M is on the scientific advisory board of Antipodean Pharmaceuticals, Inc. All other authors declare that they have no competing interests.

Author contributions

S.-Y.P., O.S.K, and R.S.R. designed and wrote the paper; O.S.K and S.-Y.P. performed experiments and analyzed data; R.H.I.A. and J.R.H. provided SMFAs; M.P.M. provided MitoQ and contributed to the revision of the article. All authors have approved the final version of the manuscript, agree to be accountable for all aspects of the work and quality for authorship.

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FIGURE LEGENDS

Figure 1. The relative abundance of skeletal muscle feed artery proteins from the electron transport chain (ETC) of young subjects and old subjects with and without MitoQ. The ETC protein expression was normalized by β-actin protein expression. Data are expressed as mean ± SE. n = 10 young and 16 old subjects. * Significantly different from young, $P<0.05$.

Figure 2. The vasodilatory dose response curves of skeletal muscle feed arteries from young subjects and old subjects with and without MitoQ evoked by flow, acetylcholine (ACh), and sodium nitropruside (SNP). Data are expressed as mean ± SE. n = 10 young and 16 old subjects. * Significantly different from old, $P<0.05$.

Figure 3. Mitochondria-specific superoxide production and manganese superoxide dismutase **(MnSOD) protein expression in skeletal muscle feed arteries of young subjects and old subjects with and without MitoQ.** Superoxide levels were assessed utilizing the mitochondrial-specific superoxide spin trap mitoTempo-H and electron paramagnetic resonance (EPR) spectroscopy. The EPR signal was expressed as the area under the curve (AUC) in arbitrary units and representative spectra are inlayed. The MnSOD protein expression was normalized by β-actin protein expression. Data are expressed as mean ± SE. n = 8 young and 8 old subjects for EPR and n = 8 young and 10 old subjects for immunoblotting. MnSOD expression of young, old, old + MitoQ. * Significantly different from young and old+MitoQ, $P<0.05$; ǂ Significantly different from young, $P<0.05$.

Figure 4. The relative abundance of proteins for endothelial NOS (eNOS) and phosphorylated (p-) eNOS at Ser1177 from skeletal muscle feed arteries of young subjects and old subjects with and without MitoQ. Data are expressed as mean ± SE. n = 8 young and

10 old subjects. * Significantly different from young and old+MitoQ, P<0.05; ǂ Significantly different from young, *P*<0.05.

Figure 5. The vasodilatory dose response curves of skeletal muscle feed arteries from young subjects and old subjects both with and without MitoQ and with and without nitric oxide synthase blockade (L-NMMA) evoked by both flow and acetylcholine (ACh). Data are expressed as mean ± SE. n = 8 young and 10 old subjects. * Significantly different from young and old+MitoQ, P < 0.05; † Significantly different from all other groups and conditions, P< 0.05.

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Table 1. Subject characteristics.

	Young (n=18)	Old (n=26)
Age (year)	32±6	75±7 *
Sex (male/female, n)	10/8	15/11
Height (cm)	175±15	165±12
Body mass (kg)	74±13	81±10
BMI (kg m^{-2})	21±7	27±7
Systolic blood pressure (mmHg)	116±7	126±9
Diastolic blood pressure (mmHg)	78±5	81±9
Glucose (mg dl^{-1})	110.8±9.2	108±5.2
Blood urea nitrogen (mg dl^{-1})	17.4±5.0	16.8±6.4
Creatinine (mg dl^{-1})	0.9±0.7	1±0.9
Albumin (g dl^{-1})	4.2±0.6	4.2±0.7
Lactate dehydrogenase (U L^{-1})	505.4±40.1	503±47.3
Hemoglobin (g dl^{-1})	15.5±1.2	14.3±1.5
White blood Cells (thousands per microliter, K ul^{-1})	4.9±2.1	7.7±1.4
Red blood Cells (millions per microliter, M ul^{-1})	5.2±1.3	4.8±1.5
Platelets (K ul^{-1})	255.9±21.1	240±27.2
Hematocrit (%)	41.4±3.1	40±5
Lymphocytes (%)	34.3±3.3	33±8.5
Monocytes (%)	8.6±1.6	8.1±2.5
Medications (Users/n)		
Diuretics	0/18	2/26
Angiotensin- converting enzyme inhibitors	0/18	2/26
Diabetic drugs	0/18	3/26
Statins	0/18	2/26

Data are expressed as mean ± SE or number of subjects (of the total number; *n*).

*Significantly different from young subjects, $P<0.05$

Figure 1.

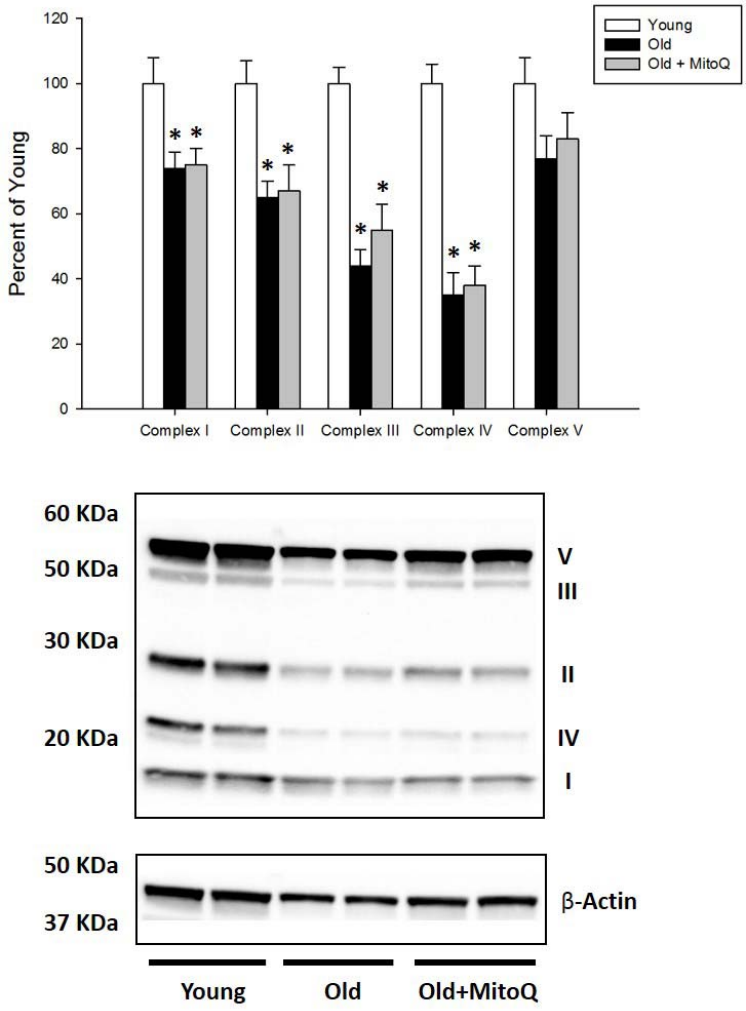
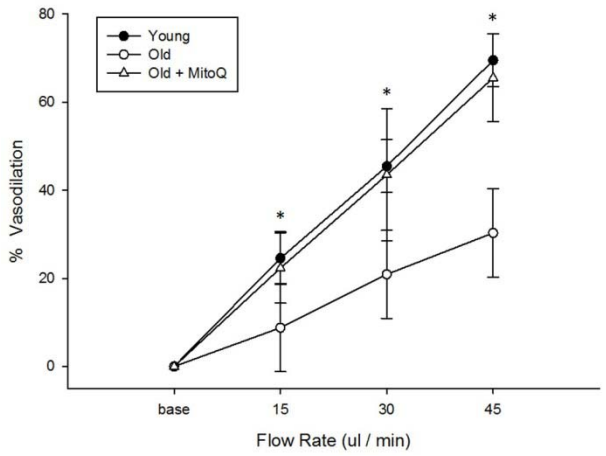
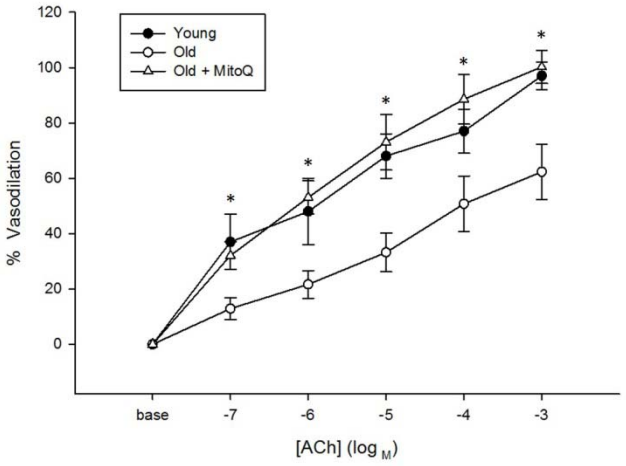

Young
Old
Old + MitoQ
Percent of Young
120
100
80
60
40
20
0
Complex I
Complex II
Complex III
Complex IV
Complex V
60 KDa
50 KDa
30 KDa
20 KDa
V
III
II
IV
I
50 KDa
37 KDa
β-Actin
Young
Old
Old+MitoQ

Figure 2.

A)


Young
Old
Old + MitoQ
% Vasodilation
0
20
40
60
80
base
15
30
45
Flow Rate (ul / min)
*

B)


Young
Old
Old + MitoQ
% Vasodilation
0
20
40
60
80
100
120
base
-7
-6
-5
-4
-3
[ACh] (log M)
*

C)

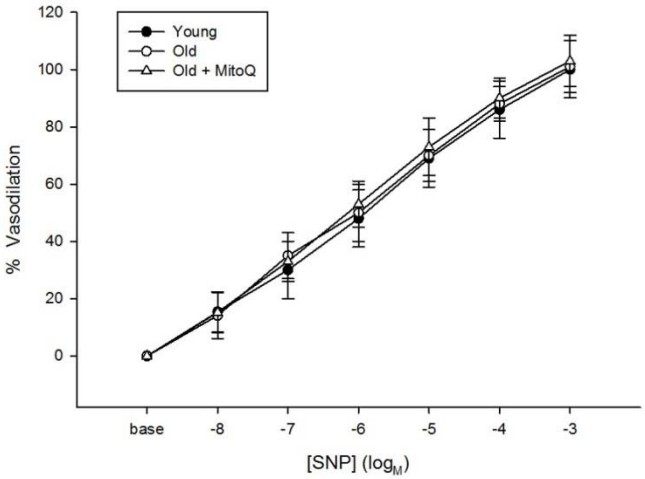
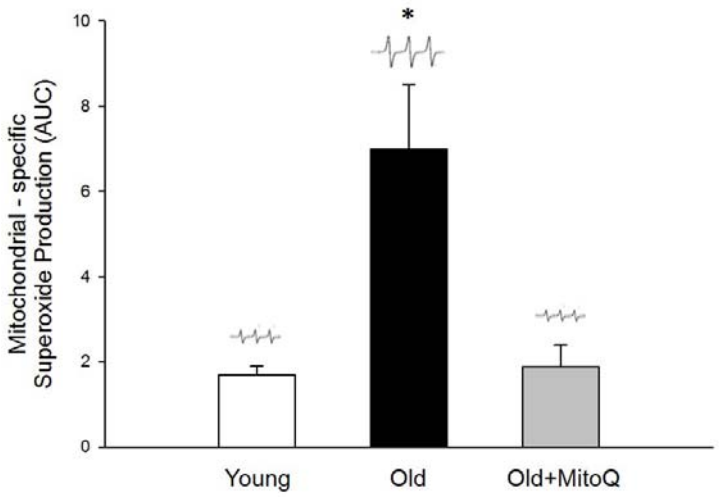

Young
Old
Old + MitoQ
% Vasodilation
0
20
40
60
80
100
120
base
-8
-7
-6
-5
-4
-3
[SNP] (log M)

Figure 3.

A)


Mitochondrial - specific Superoxide Production (AUC)
10
8
6
4
2
0
*
Young
Old
Old+MitoQ

B)

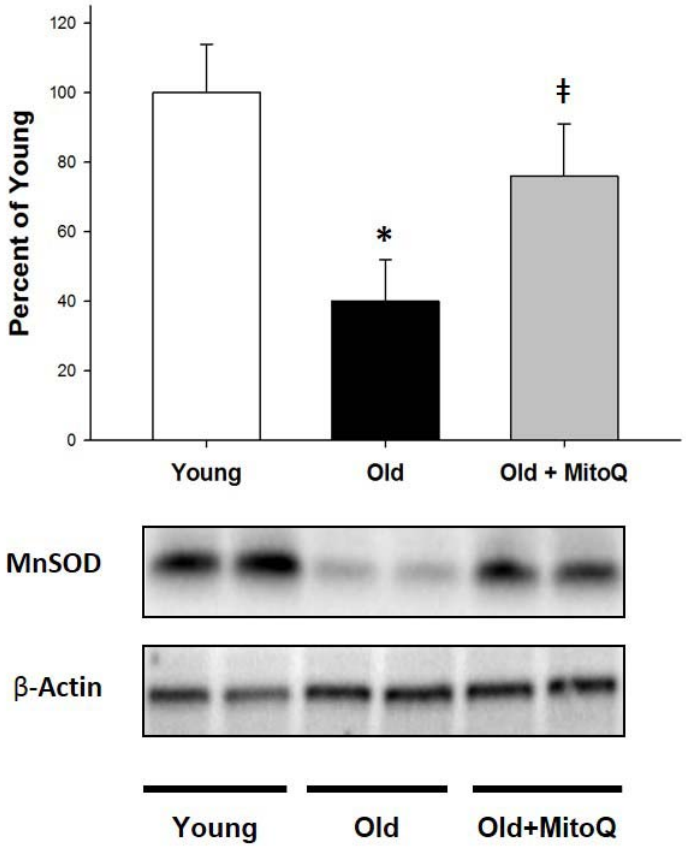

Percent of Young
120
100
80
60
40
20
0
*
‡
Young
Old
Old + MitoQ
MnSOD
β-Actin
Young
Old
Old+MitoQ

Figure 4.

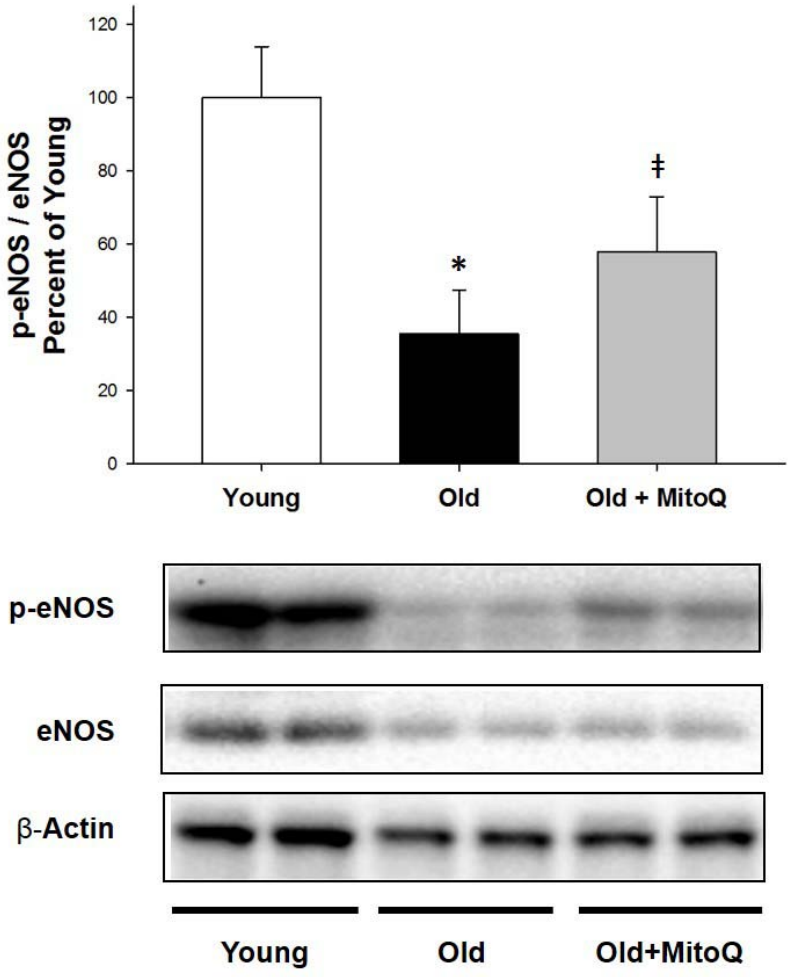
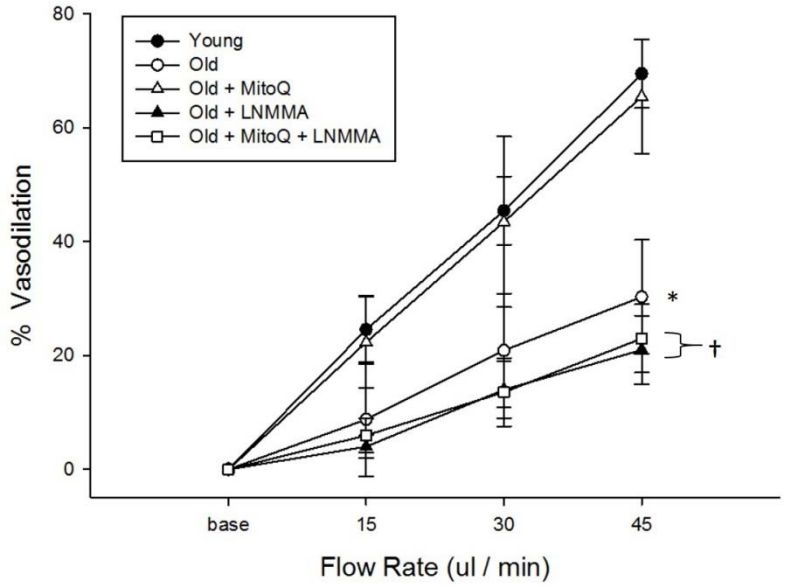
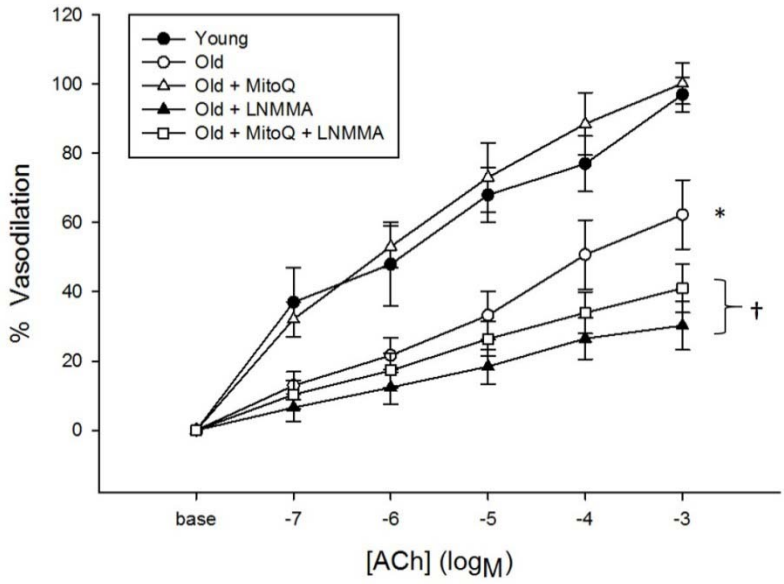

p-eNOS / eNOS
Percent of Young
120
100
80
60
40
20
0
*
‡
Young
Old
Old + MitoQ
p-eNOS
eNOS
β-Actin
Young
Old
Old+MitoQ

Figure 5.

A)


Young
Old
Old + MitoQ
Old + LNMMA
Old + MitoQ + LNMMA
% Vasodilation
80
60
40
20
0
base
15
30
45
Flow Rate (ul / min)
*
†

B)


Young
Old
Old + MitoQ
Old + LNMMA
Old + MitoQ + LNMMA
% Vasodilation
120
100
80
60
40
20
0
base
-7
-6
-5
-4
-3
[ACh] (logM)
*
†